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Effect of propentofylline (HWA 285) on extracellular purines and excitatory amino acids in CA1 of rat hippocampus during transient ischaemia

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1 The adenosine uptake blocker propentofylline (HWA 285) has previously been shown to protect hippocampal CA1 pyramidal cells from ischaemia-induced delayed neuronal death. The influence of propentofylline, on the extracellular concentrations of purines, aspartate and glutamate in the CA1 of the rat hippocampus during transient forebrain ischaemia was investigated.

2 Twenty min of ischaemia was induced by four-vessel occlusion in Wistar rats, extracellular compounds were sampled by use of microdialysis and EEG was recorded by a tungsten electrode attached to the dialysis probe.

3 Propentofylline ($10\,mg\,kg^{-1}$ i.p.) did not influence the basal levels of any of the compounds in the hippocampal dialysates.

4 The EEG became isoelectric within 20 s after induction of ischaemia.

5 Extracellular adenosine, inosine, hypoxanthine, aspartate and glutamate increased several fold during ischaemia and remained elevated during early reflow. Within 2 h of reperfusion the concentration of all compounds was normalized. Xanthine increased upon reperfusion and remained elevated after 2 h.

6 Propentofylline ($10\,mg\,kg^{-1}$ i.p.) administered 15 min before ischaemia significantly enhanced the ischaemia-evoked increase of adenosine but attenuated the increases of the other purine catabolites and of glutamate.

7 In separate *in vitro* experiments, propentofylline did not inhibit adenosine deaminase activity.

8 The present data show that propentofylline enhances extracellular adenosine and lowers extracellular glutamate *in vivo* during ischaemia. These findings may be important in relation to the neuroprotective properties of propentofylline.

Introduction

Previous experiments utilizing the microdialysis technique have revealed major changes in the extracellular concentrations of neuroactive compounds in the brain during cerebral ischaemia. There is a massive increase of adenosine and its catabolites (Hagberg *et al.*, 1987) and of the excitatory amino acid (EAA) neurotransmitters glutamate and aspartate (Benveniste *et al.*, 1984; Hagberg *et al.*, 1985).

The probable pathophysiological role of glutamate and aspartate as potent neurotoxins during brain ischaemia has been emphasized in recent years (Jörgensen & Diemer, 1982; Meldrum, 1985). Deafferentation of the EAA input to the hippocampus (Wieloch *et al.*, 1985; Johansen *et al.*, 1987) and administration of EAA-receptor antagonists of the N-methyl-D-aspartate (NMDA) type (Gill *et al.*, 1987; Park *et al.*, 1988; Swan *et al.*, 1988) offer neuroprotection in some animal models of ischaemia.

Adenosine may also act as a neuroprotective agent in brain ischaemia by inhibiting the ischaemia-induced release of excitotoxic amino acids and, possibly, also by improving the brain circulation (see Dragunow & Faull, 1988 for references). For example, recent studies demonstrate that the well known adenosine receptor antagonist theophylline aggravates (Rudolphi *et al.*, 1987) and metabolically stable adenosine analogues attenuate ischaemic brain damage (Evans *et al.*, 1987; von Lubitz *et al.*, 1988). Unfortunately, it is difficult to administer adenosine agonists systemically because of their strong cardiovascular side-effects such as hypotension and cardiodepression (Fredholm & Sollevi, 1986). Instead it could be possible to augment the purported protective effects of adenosine by agents that decrease adenosine inactivation. Such an agent would increase the chance of endogenously formed adenosine interacting with its A_1- and/or A_2-receptors at the exterior surface of the cell membrane and the action would be restricted to such areas where adenosine levels are increased, thereby limiting the risks of untoward effects. The classical, potent adenosine-uptake inhibitor dipyridamole cannot be used in the case of brain ischaemia because of its poor permeability through the blood brain barrier, but there is a wide variety of chemically diverse compounds, although of lower potency, which have also been demonstrated to inhibit adenosine-uptake (Phillis & Wu, 1982; Fredholm & Lindström, 1986). Amongst them the novel xanthine derivative propentofylline (HWA 285) has recently been found to prevent ischaemia-induced calcium loading and subsequent necrosis of hippocampal neurones in Mongolian gerbils (DeLeo *et al.*, 1987; 1988a,b).

It was therefore the aim of the present study to examine the influence of this drug on the extracellular concentration of adenosine and its catabolites in the hippocampus before, during and after transient forebrain ischaemia. As previous studies imply an interaction between the adenosine- and EAA-systems (Dolphin & Archer, 1983; Corradetti *et al.*, 1984), we also studied the effect of propentofylline on the ischaemia-induced release of the amino acids glutamate and aspartate.

Since the effects of adenosine uptake inhibitors on extracellular concentrations of adenosine and its catabolites are essentially the same as those produced by inhibitors of the

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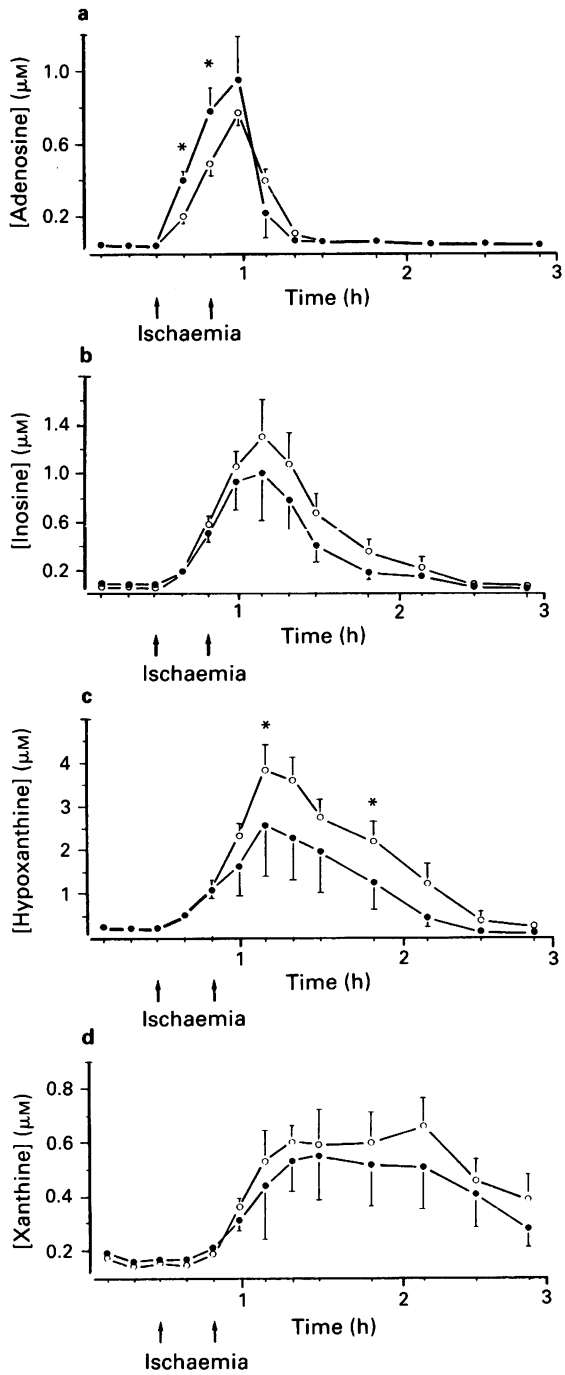

Figure 1 Concentration of (a) adenosine, (b) inosine, (c) hypoxanthine and (d) xanthine before, during and after 20 min of bilateral forebrain ischaemia in the dialysate of rat hippocampus (CA1-area) in controls (○) and after i.p. administration of propentofylline (HWA 285) 15 min before ischaemia (●). In both groups the concentration of adenosine, inosine and hypoxanthine increased multifold during ischaemia and remained elevated during early reflow. Xanthine increased upon reperfusion. The treatment with propentofylline augmented the increase of adenosine during ischaemia, whereas the increase of the other purines was attenuated. Data are mean and vertical lines show s.e.mean; $n = 8$ per group. * Indicates significant difference between control and treated group ($P < 0.05$, Mann-Whitney).

adenosine degrading enzyme adenosine deaminase (ADA), we also tested whether propentofylline had ADA inhibiting properties.

Methods

Microdialysis experiments

Male Wistar rats (300–350 g, $n = 16$) were anaesthetized with

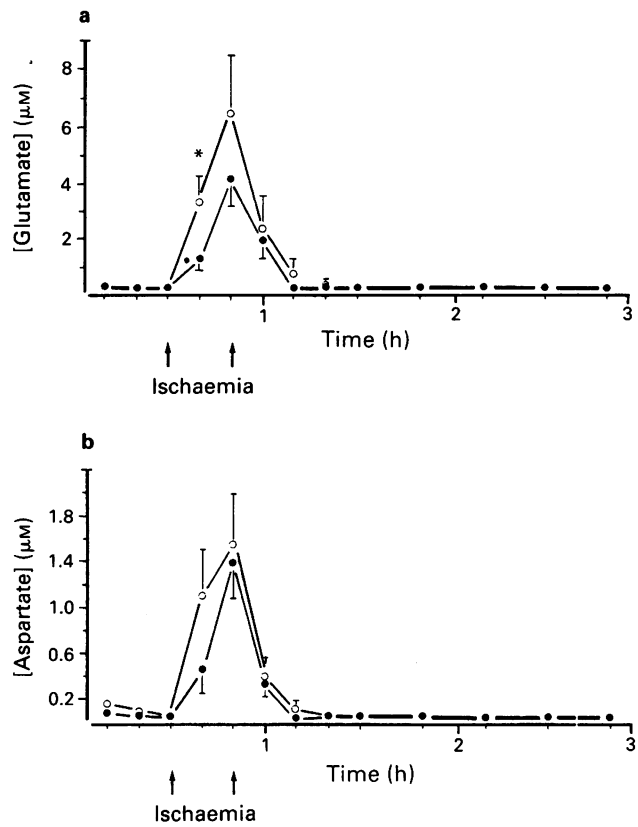

Figure 2 Concentrations of (a) glutamate and (b) aspartate before, during and after 20 min of bilateral forebrain ischaemia in the dialysate of rat hippocampus (CA1-area) in controls (○) and after i.p. administration of propentofylline (HWA 285) 15 min before ischaemia (●). In both groups the concentration of glutamate and aspartate rapidly increased during ischaemia. In the propentofylline-treated group the increase in glutamate concentration was attenuated during the ischaemic period with a significantly lower level at 10 min of ischaemia, and the mean aspartate concentration showed a tendency towards lower levels. Data are mean and vertical lines show s.e.mean; $n = 8$ per group. * Indicates significant difference between control and treated group ($P < 0.05$, Mann Whitney).

methohexitone (Lilly & Co., 60 mg kg^{-1} i.p.) and both vertebral arteries were permanently occluded by electrocauterization (Pulsinelli & Brierley, 1979). The animals were fasted overnight, reanaesthetized the following day with halothane (5% for induction and 0.7% for maintenance) in 2:1

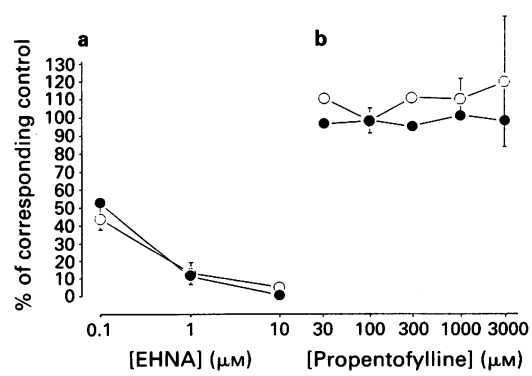

Figure 3 Effect of (a) erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and (b) propentofylline on adenosine deaminase activity (ADA). Close to 80% of the [^{3}H]-adenosine was converted to [^{3}H]-inosine by the pure adenosine deaminase during the 10 min incubation (3 separate experiments in duplicate; ●). The brain homogenate (from 9 mg cortical tissue) converted 0.5 nmol [^{3}H]-adenosine to inosine in 10 min (2 separate experiments in duplicate or triplicate; ○). In contrast to EHNA, propentofylline did not inhibit ADA. Each point shows the mean and vertical lines indicate s.d.

N_2O and O_2 and intubated with a polyethylene tubing (Intramedic PE 240, Clay Adams, Parsippany, NJ, U.S.A.). The carotid arteries were exposed and the head of the rat was put in a stereotaxic frame. The body temperature was kept at 37°C with a thermistor-regulated heating pad. The temperature was measured in the temporal muscle of the head. The skull surface was exposed and a hole was drilled through the cranium. A microdialysis electrode (Sandberg *et al.*, 1986) was implanted into the CA1 region of the hippocampus (2.2 mm lateral, 3.8 mm dorsal to bregma, and the window of the dialysis membrane 1.4–2.9 mm down from the cortical surface). The electroencephalogram (EEG) was measured continuously with a tungsten electrode attached to the dialysis probe. The dialysis electrode was perfused (2.5 μl min^{-1}) with an unbuffered Ringer solution (NaCl 145 mM, KCl 3 mM, Ca_2Cl 1.2 mM) and samples were taken every 10 min until 40 min of reperfusion, followed by another 4 samples at 20 min intervals. The dialysates were frozen and stored at −80°C until analysed. A steady state period of 2 h was followed by three 10 min samples ('basal levels'). Then either saline ('control animals') or 10 mg kg^{-1} propentofylline (HWA 285, 1,5-oxohexyl-3-methyl-7-propylxanthine, Hoechst AG Werk Kalle-Albert, Wiesbaden, F.R.G.) dissolved in saline was administered intraperitoneally. Samples were then taken for another 2 h followed by a new intraperitoneal injection of saline or propentofylline (10 mg kg^{-1}). Fifteen min later the carotid arteries were clamped for 20 min and dialysis perfusion was maintained during the ischaemic period and for 2 h of postischaemic reperfusion.

As previously described the dialysates were analysed by liquid chromatography and u.v. detection for purine catabolites (Hagberg *et al.*, 1987) and by liquid chromatography and fluorescent detection for amino acids (Lindroth *et al.*, 1985).

The experimental data of the control group and the drug-treated group were compared by Mann-Whitney unpaired two-tailed test. $P < 0.05$ was accepted as being significant.

Measurement of adenosine deaminase activity

Adenosine deaminase (ADA) activity was determined essentially as described by Fredholm *et al.* (1984). In brief, the ADA (either 0.01 u purified adenosine deaminase from calf intestine Boehringer, Mannheim or approximately 1.4 mg of a crude 2000 *g* supernatant of rat cortex) was incubated for 10 min at 30°C with [^{3}H]-adenosine (Radiochemical Centre, Amersham; 0.1 μCi; 5–10 nmol) and 20 nmol inosine in Tris buffer pH 7.4 containing EDTA. The reaction was stopped by formic acid and the sample was put on a 2.5 ml column of SP-sephadex (Pharmacia); the [^{3}H]-inosine was eluted with 8 ml 0.1 M formic acid and the unaltered [^{3}H]-adenosine with 8 ml 0.1 M sodium formate pH 5.5.

Results

Microdialysis experiments

In both experimental groups the hippocampal EEG activity ceased within 20 s after the carotid arteries had been clamped and remained isoelectric throughout the ischaemic period. This confirms the ischaemic state of the rat hippocampus. By 2 h of reperfusion the EEG activity had recovered almost completely. Propentofylline did not influence the EEG pattern.

The changes of adenosine, inosine, hypoxanthine and xanthine levels in the hippocampus dialysate are presented in Figure 1.

In the untreated control group they followed the same pattern as previously described by Hagberg *et al.* (1987). The adenosine concentration in the dialysate increased nineteen fold during ischaemia and early reperfusion. The peak level was reached at 10 min of reperfusion. Dialysate inosine and hypoxanthine concentrations also increased during ischaemia and reperfusion, reaching their maximum values at 20 min of recirculation, whereas the xanthine concentration remained unchanged during ischaemia but increased upon reflow. Pretreatment with propentofylline did not influence the basal levels of adenosine and its catabolites in the hippocampus dialysate. However, during ischaemia and in the early reperfusion period the adenosine concentration was increased in the propentofylline-treated group. This effect was statistically significant at 10 and 20 min of ischaemia (50% and 23%, respectively). The adenosine level remained above the values of the control group until 10 min of reperfusion and returned to pre-ischaemic basal values within the following 20 min. The purine catabolites showed a clear overall trend towards lower levels in the propentofylline-treated group throughout the reperfusion period. However, this was statistically significant only for hypoxanthine at 20 and 60 min of reperfusion (−33%, −44%).

The changes in the dialysate concentration of glutamate and aspartate are shown in Figure 2. In both experimental groups their concentrations increased rapidly during ischaemia, reaching maximal values at the end of the ischaemic period, and returned to pre-ischaemic levels within the first 20 min of reperfusion. Pretreatment with propentofylline led to an overall reduction of the glutamate concentration over the entire ischaemic period with a significantly lower level at 10 min of ischaemia (−75%) when compared with the untreated group. Although not statistically significant, the mean aspartate level was lower at 10 min of ischaemia in the hippocampal dialysates of the propentofylline-treated rats.

Effect on adenosine deaminase activity

Propentofylline did not inhibit either ADA activity in brain homogenate or the commercially obtained purified enzyme. The ADA-inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) on the other hand was as active as previously described (Figure 3) (see Fredholm *et al.*, 1984).

Discussion

In both experimental groups the transient forebrain ischaemia produced similar changes of adenosine, inosine, hypoxanthine and xanthine in dialysates from the hippocampus as previously described for the rat striatum (Zetterström *et al.*, 1982; Hagberg *et al.*, 1987). The increase in the adenosine concentration and the lowering of the level of adenosine catabolites in the hippocampus dialysate which was produced by pretreatment with propentofylline are consistent with the properties of either an adenosine transport inhibitor such as dipyridamole or an inhibitor of ADA. However, we showed in the present study that propentofylline, in contrast to a known ADA-inhibitor, EHNA, did not inhibit ADA *in vitro*. *In vivo* EHNA markedly lowered brain dialysate inosine levels, leaving the adenosine concentration virtually unaffected (Zetterström *et al.*, 1982). We therefore exclude the possibility that propentofylline acts *in vivo* as an ADA inhibitor.

High affinity saturable and reversible adenosine transport sites have been characterized by binding experiments and localized by *in vitro* autoradiography in the brain of various animal species (Marangos *et al.*, 1985; Nagy *et al.*, 1985; Deckert *et al.*, 1987; 1988). In the cerebral cortex they are heterogeneously distributed (Nagy *et al.*, 1985; Deckert *et al.*, 1988). However, their distribution has been shown to differ markedly from that of A_1-receptors. Recent findings suggest that microvessels have a much higher density of the nucleoside transporter than parenchymal cells in the cerebral cortex (Kalaria & Harik, 1988).

To our knowledge we provide here the first experimental evidence of a drug-induced increase of extracellular adenosine concentration in the brain during ischaemia. *In vitro* studies have demonstrated that propentofylline is a moderately strong

inhibitor of adenosine uptake in human erythrocytes (Porsche, 1982a; Fredholm & Lindström, 1986) and in bovine microvessels (Stefanovich, 1983). Our data indicate that propentofylline acts *in vivo* as a nucleoside transport inhibitor in the ischaemic brain in a dose which has previously been shown to attenuate ischaemic brain damage (DeLeo *et al.*, 1987). With the cortical cup technique, Phillis *et al.* (1989) recently obtained similar effects with the adenosine transport inhibitor dipyridamole in the hypoxic rat brain. When given systemically ($0.5\,mg\,kg^{-1}$ i.v.) the drug enhanced the hypoxia-induced increase of adenosine concentration and attenuated the increases of inosine, hypoxanthine and xanthine in rat cerebral cortex interstitial fluid without influencing the basal concentration of these purines. These findings are somewhat surprising because dipyridamole does not apparently pass the blood brain barrier (Sollevi, 1986). The authors explained their results by suggesting that dipyridamole may be able to increase brain interstitial fluid adenosine by blocking its reuptake into capillary endothelial cells. However, this requires experimental confirmation. Another nucleoside transport inhibitor, soluflazine, was ineffective in this study when administered systemically or topically, although this drug has anticonvulsant and sedative effects (Wauquier *et al.*, 1987; Ashton *et al.*, 1988) like adenosine analogues.

The administration of some adenosine transport inhibitors can mimic the pharmacological effects of adenosine in the brain (Phillis *et al.*, 1989). This is particularly pronounced in situations where there is an increase in endogenous adenosine formation, such as hypoxia/ischaemia or seizures. This may lead to a reinforcement of the possible protective role of endogenous adenosine as a 'retaliatory metabolite' (see Dragunow & Faull, 1988 for review). The reduction of the ischaemia-induced increase of extracellular glutamate and aspartate by propentofylline, observed in the present study, may be interpreted as an indirect effect of the reinforcement of the inhibitory effect of endogenous adenosine on the release of these two EAAs (Dolphin & Archer, 1983; Corradetti *et al.*, 1984; Fastbom & Fredholm, 1985). During the ischaemia period extracellular adenosine is present in a concentration of approximately 20–200 μM, i.e. a concentration range known to attenuate the release of EAAs *in vitro* (Corradetti *et al.*, 1984). However, we have no evidence for a direct effect of propentofylline on glutamate or aspartate release. In addition propentofylline, like adenosine and some of its metabolically stable analogues, increases cerebral blood flow (Grome *et al.*, 1985) and inhibits platelet aggregation (Seiffge & Kremer, 1986).

Even though many aspects of the pharmacology of propentofylline can be explained by nucleosine transport inhibition, it has also been shown to be a relatively potent inhibitor of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterases in various tissues (Stefanovich, 1985; Nagata *et al.*, 1985). The resulting increase of cyclic AMP could contribute to the vasodilator effects and the antithrombotic activity of propentofylline. It may also explain the observed supra-additive effects of the drug and high concentrations of adenosine on cyclic AMP accumulation in rat hippocampal slices (Fredholm & Lindström, 1986). In addition, there may be yet other actions of the compound that account for its inhibitory potency against kainic acid-induced neuroexcitation (Porsche, 1982b) and the reduction of ischaemia-evoked release of glutamate and aspartate.

In conclusion, we have demonstrated that pretreatment with propentofylline, a novel xanthine derivative that can act as an adenosine uptake inhibitor, reinforces the ischaemia-induced increase of adenosine and attenuates the release of the potentially excitotoxic amino acid glutamate in the extracellular space of the hippocampus. These findings may be important in relation to the previously described neuroprotective effects of propentofylline against ischaemic hippocampal damage in Mongolian gerbils.

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